

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: G01N 33/68, C07K 14/79	A1	(11) International Publication Number: WO 95/04932 (43) International Publication Date: 16 February 1995 (16.02.95)
(21) International Application Number: PCT/SE94/00684 (22) International Filing Date: 11 July 1994 (11.07.94) (30) Priority Data: 9302573-2 6 August 1993 (06.08.93) SE (71) Applicant (for all designated States except US): BIOLIN MEDICAL AB [SE/SE]; P.O. Box 7395, S-103 91 Stockholm (SE). (72) Inventor; and (75) Inventor/Applicant (for US only): JEPPSSON, Jan, Olof [SE/SE]; Ärtholmsvägen 198, S-216 20 Malmö (SE). (74) Agent: MAGNUPATENT AB; P.O. Box 6207, S-200 11 Malmö (SE).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD). Published <i>With international search report.</i> <i>In English translation (filed in Swedish).</i>
(54) Title: QUANTITATION OF CARBOHYDRATE DEFICIENT TRANSFERRIN IN HIGH ALCOHOL CONSUMPTION BY HPLC (57) Abstract <p>A method for separation and quantitation of carbohydrate deficient transferrin (CDT) in order to decide a person's alcohol consumption during the most recent time. Lipoproteins are precipitated in blood serum, saturated with iron, and then the sample is centrifuged after a short storage period. The supernatant of the centrifuged mixture is then diluted with water. Subsequently the mixture is injected to an ion exchange column, when a saltgradient is used for separation of the isoforms of transferrin. A chromatogram is developed by means of HPLC and a filter for absorbance at 460 nm. Should the chromatographic profile show increased values of CDT (*, **), more than 0.8 % of total transferrin, it is a highly specific marker for heavy alcohol consumption.</p> <div data-bbox="1182 1058 1396 1806"><p>8 10 12 14 16 TIME (MIN)</p></div>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MD	Republic of Moldova	TJ	Tajikistan
DE	Germany	MG	Madagascar	TT	Trinidad and Tobago
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain	MN	Mongolia	US	United States of America
FI	Finland			UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

QUANTITATION OF CARBOHYDRATE DEFICIENT TRANSFERRIN IN HIGH ALCOHOL CONSUMPTION BY HPLC

5

The invention relates to a method for separation and quantitation of carbohydrate deficient transferrin (CDT) in order to decide a person's alcohol consumption.

10 Alcohol causes extensive damage to its victims and their families and significant costs for society through its associated morbidity and mortality. Early recognition and treatment have shown to be beneficial for the individual and cost effective for society. Sensitive, specific, rapid and inexpensive methods for identifying the individuals at risk
15 of complications to drinking in different populations are needed. Numerous procedures designed to detect heavy drinkers have been developed during the past thirty years. Conventional laboratory tests such as γ -glutamyltransferase (γ -GT), mean corpuscular volume (MCV), aspartate or alanine aminotransferases (AST or ALT), α -lipoproteins and
20 ferritin have been used for many years as biochemical markers of alcohol abuse, but have low diagnostic sensitivity and specificity. A qualitative change in isoforms of transferrin in cerebrospinal fluid and serum of patients with alcohol related cerebellar tremor has been reported. The isoforms related to alcohol abuse contained less sialic
25 acid than other isoforms and can therefore be distinguished according to charge. Several techniques for separation of isoforms have been introduced but they are generally laborious, non-quantitative and expensive. Chromatofocusing, disposable mini-columns combined with RIA (radioimmunoassay) and electrofocusing followed by immunofixation,
30 Western blotting or zone immunoelectrophoresis are the available techniques.

Transferrin, the iron-transporting protein in blood, is a glycoprotein with two bi- or tri-antennary carbohydrate chains, each terminated with two or three sialic acids (N-acetylneuraminic acid), respectively. Isoelectric focusing separates normal transferrin with high resolution into isoforms depending on iron saturation, content of sialic acid (SA) or amino acid substitutions. After complete iron saturation (2 Fe atoms per molecule) transferrin normally separates into 4 isoforms named after their approximate isoelectric point (pI) pI 5.2 (5 SA), pI 5.4 (4 SA, major fraction), pI 5.6 (3 SA) and pI 5.7 (2 SA). Small amounts of transferrin with pI 5.6 and 5.7 are present in normal serum. The isoform which markedly increases in serum from alcoholics is pI 5.7. The pI 5.7 fraction is normally less than 0.8 % of total transferrin, but may be increased more than tenfold after heavy alcohol consumption. After excessive drinking and additional pI 5.9 fraction (0 SA) may appear. The pI 5.7 and pI 5.9 fractions represent carbohydrate deficient transferrin (CDT).

The purpose of this invention is to develop an HPLC (high performance liquid chromatography) method suitable for routine laboratories to identify subjects at high risk for alcohol dependence and to evaluate its sensitivity and specificity for detecting heavy alcohol consumption in defined populations. The object is to present a method which is suitable for clinical standard procedures and which specifically measures the concentration of CDT, which is a suitable biochemical marker for heavy alcohol consumption during the preceding weeks. The object is met with a method according to the main Claim below.

The method of the invention is described in greater detail below by means of some Figures, in which:

- Fig. 1 shows chromatograms for transferrin isoforms from samples from individuals with varying alcohol consumption;
- Fig. 2 shows how the concentration of CDT decreases after finished drinking for four heavily intoxicated patients;
- Fig. 3 shows, in tabular form, the salt gradient used when separating transferrin isoforms; and
- Fig. 4 shows CDT levels in sera of individuals with different alcohol consumption.

Samples were prepared in that fresh serum or serum frozen at -20°C for less than 6 months was saturated with iron by addition of $25\text{ }\mu\text{l}$ of NaHCO_3 (500 mmol/l) and $18\text{ }\mu\text{l}$ FeCl_3 (10 mmol/l) per ml serum. After mixing and storage at $+8^{\circ}\text{C}$ overnight, the lipoproteins were precipitated by adding $10\text{ }\mu\text{l}$ of Dextranesulfate (10% (w/v)) and $50\text{ }\mu\text{l}$ of CaCl_2 (1 mol/l) per ml serum. This mixture was stored for 30-60 min. at $+8^{\circ}\text{C}$ and then centrifuged at $10\,000 \times g$ for 10 min. The supernatant was diluted fivefold with water and transferred to an HPLC autoinjector.

The transferrin isoforms were separated on an ion exchange column, Mono Q®HR 5/5 (Pharmacia Biotechnology, Sweden) from other serum proteins by a saltgradient for 32 min. including regeneration. Starting buffer (A) was Bis Tris 20 mmol/l pH 6.2. Buffer B was buffer A plus NaCl 350 mmol/l at the same pH. Solution C, NaCl 1 mol/l , was used for regeneration. Before use all solutions were degassed and filtered through a $0.45\text{ }\mu\text{m}$ pore - size filter. Samples of $200\text{ }\mu\text{l}$ were injected, the flow rate was maintained at 1 ml/min . providing the gradient profile shown in the table of Fig. 3.

The HPLC system used consisted of pump No. 2941 (Pharmacia Biotechnology, Sweden), a Jasco 870 UV detector equipped with a 460 nm filter and a 10 mm flow cell together with a wolfram lamp. The system contained an autoinjector, Waters WISP 715, with a cooling system for 96
5 samples. An Shimadzu CR 5A integrator was used for calculating the peak areas according to the valley-valley mode.

Transferrin isoforms have pI:s between approximately 5.2-5.9. Optimal separation of the most cathodal isoforms was obtained at pH 6.2. Nevertheless, acceptable results have been obtained also with buffer solu-
10 tions having pH values between 6.0 and 6.4. However, for the best result pH should be between 6.1 and 6.3, and most preferably pH should be 6.2. The addition of NaHCO₃ together with FeCl₃ gives an optimal stable iron saturation. Precipitation of lipoproteins improves the
15 separation of the pI 5.9 isoform which can be hidden under heavy β -lipoprotein fractions in some patients. Fasted samples with lower levels of lipoprotein cannot be used in working with alcoholics on ambulatory bases. Fig. 1 illustrates chromatograms after HPLC from heavily, medium and normal drinking individuals, where the pI 5.9
20 isoform also appears in the first pattern. The absorbance of the Fe-transferrin complex at 460 nm is approximately 1/10 of the 280 nm absorbance, but is highly specific for the transferrin fractions. The amount of CD-transferrin, pI 5.7 represents only 0.2-0.8 % (mean \pm 2 S.D.) of the total transferrin in teetotallers and occasional drinkers.
25 This value was slightly dependent on the method of integration of the chromatography profile. Slightly higher values were found using baseline integration, but the valley-valley method was more reproducible.

In Fig. 1, A represents a sample from an individual with the consumption of 300 g alcohol/24 h, B represents a sample from an individual
30 having consumed 70 g alcohol/24 h, and C indicates a normal pattern.

The shadowed areas show CDT, i.e. transferrin isoforms having pI 5.7 (*) and pI 5.9 (**). The other peaks of the chromatograms represent, from the right, pI 5.2, 5.4 and 5.6, respectively. It is obvious from the chromatograms that the dominating fraction is pI 5.4. It is further
5 obvious that the values of CDT are increased after heavy alcohol consumption. The absorbance of the Fe-transferrin complex was measured at 460 nm.

The half-life of CD-transferrin was studied by measuring the CDT pI 5.7
10 of four heavily intoxicated patients sequentially for 15 days hospitalisation at which time no relapse of alcohol abuse occurred. The total transferrin concentration was measured in each sample (g/l) and CDT was calculated and given as mg/l in a semilogarithmic diagram (Fig.2). Total transferrin concentration increased with time in some patients during
15 the hospitalisation. The half-life of each curve was read from its linear slope. There was a small difference between individual patients and the mean $T_{1/2}$ was estimated to be 9.5 ± 1 days.

CDT values found in samples from teetotallers and occasional drinkers
20 (laboratory staff) showed a normal distribution and were consistently below 1 % using valley-valley integration (Fig. 4). Among 284 men from the general city population with a previous record of high γ -GT values 20 % declared an alcohol consumption of 40-70 g/24 h. In these men the sensitivity of CDT was 55 % and the specificity 91 % using the cut off
25 level of 0.8 %. Among the heavily intoxicated drinkers (70-500 g/24 h) the sensitivity was nearly 100 %. Normalisation of CDT was seen in 84 % during aversion therapy. Some of them have not yet reached their basal CDT level. We cannot exclude moderate drinking during treatment with calcium carbide tablets.

The method gives reproducible results and can be automated for large sample series. Forty patients samples can be analyzed during 24 hours. The costs for reagents including investments for HPLC are approximately 30 % of the reagent costs for the disposable minicolumns combined with radioimmunoassay (Pharmacia Diagnostics). This technique has to be run in duplicates. Another advantage of HPLC is the visible document of the specific 460 nm absorbance which is of importance in the genetic variation of transferrin. Serum from alcoholics are often lipemic and some lipoproteins as well as other serum proteins may precipitate at pH 6.2 causing a gradual increase in column pressure. It is therefore convenient to use two columns so analyses can be performed during regeneration of one column. This approach has provided us with one year of experience (>1000 samples) with no problems. Genetic variants of transferrin are estimated to be present in about 29 % of the population. Most of them represent subtypes of the major TfC phenotype with minor changes in pI:s. They do not interfere with the chromatography pattern. Only TfBC heterozygotes and TfCD heterozygotes, frequency 1-2 ‰ in Caucasian population will interfere with the chromatography profile. In these cases it is necessary to confirm the results with isoelectric focusing in a specialized laboratory.

To confirm the method of the invention albumin was removed from serum by pretreatment with Blue-Sepharose®. Disposable columns (polypropylene 5 ml, Pierce) were packed with swollen Blue-Sepharose® C1-6B (Pharmacia Biotechnology, Sweden) corresponding to 0.2 g (dry weight). After rinsing according to the manufacture's instruction the small column was equilibrated with glycine, 100 mmol/l, pH 7.2. 100 µl of the iron-saturated and lipoprotein free supernatant was mixed with 2 µl β-mercaptoethanol 10 % (v/v) and after one hour at room temperature applied to the mini-column. Serum proteins were eluted with glycine, 100 mmol/l, pH 7.2

The first 300 μ l of the eluate was discarded, 40 μ l of the next mixed 800 μ l was used for isoelectric focusing. Each column can be used several times after regeneration with urea 6 mol/l followed by glycine 0.1 mol/l, pH 7.0.

Isoelectric focusing was performed essentially as described for α_1 -antitrypsin with the following modifications. The ampholyte mixture was equal parts of Pharmalyte[®] 4-6.5 and Pharmalyte[®] 5-6, total 1.9 ml per 30 ml gel solution. The same procedure can be performed on a smaller scale using the Phast System (Pharmacia Biotechnology, Sweden). Transferrin isoforms can be verified by immunofixation using cellulose acetate membranes impregnated with transferrin antibodies. All pathological results from HPLC (CDT >0.8%) have so far been confirmed by isoelectric focusing.

The serum transferrin concentration was estimated by electroimmunoassay using antisera from Dako (Denmark).

Our procedure gives automatically the calculated percentage of CDT from the integrator and eliminates the need for specific determination of transferrin concentration. The observation of large variation of total transferrin concentration in women with increases due to iron deficiency and oestrogen administration and highly intoxicated alcoholics (range 1.1-3.6 g/l) in this work motivates the use of percentage CDT of total amount transferrin rather than absolute quantities. The half-life of approximately 9.5 days for the pI 5.7 isoform allows evaluation of alcohol consumption during the past 1-3 weeks or verifies a successful treatment. This result is in agreement with the published half-life of 8-10 days for normal transferrin rather than the recently estimated 15 days for CDT.

When CDT is elevated it is a very specific marker for alcohol abuse and greatly superior to other currently available biological markers. In an evaluated population of middle-aged males the individuals have been
5 very thoroughly characterized and followed over 15 years. At a follow-up in 1991 the alcohol consumption was assessed by two trained nurses and blood sampled for CDT at the same occasion. We found a sensitivity of 55 % in the group that reported an alcohol consumption of more than 40 g/day. However, in the study of intoxicated individuals consuming
10 more than 70 g/day the sensitivity was near 100 %. The high specificity in the teetotallers and the occasional drinkers, near 100 % allows the conclusion that CDT has the highest specificity of the available biological markers.

CLAIMS

1. A method for separation and quantitation of CD-transferrin in blood serum, c h a r a c t e r i z e d in that the blood serum is
5 saturated with iron and that isoforms of transferrin are separated from other serum proteins in an ion exchange column using a salt-gradient, a chromatogram is developed by means of high performance liquid chromatography (HPLC) which chromatogram shows the relation between isoforms of transferrin with different pI values.
10
2. The method as claimed in Claim 1, c h a r a c t e r i z e d in that the separation takes place in a buffer solution having a pH of between 6.0 and 6.4, and preferably between 6.1 and 6.3.
- 15 3. The method as claimed in Claim 1, c h a r a c t e r i z e d in that the separation takes place in a buffer solution having a pH of 6.2.
4. The method as claimed in Claim 1, c h a r a c t e r i z e d in
20 selective absorbance of a Fe-transferrin complex at 460 nm.
5. The method as claimed in Claim 4, c h a r a c t e r i z e d in that the chromatography profile is integrated using the valley-valley method.
25
6. The method as claimed in Claim 1, c h a r a c t e r i z e d in that the ion saturation is made by adding 25 μ l NaHCO_3 (500 mmol/l) and 18 μ l FeCl_3 (10 mmol/l) per ml serum.
- 30 7. The method as claimed in Claim 6, c h a r a c t e r i z e d in that lipoproteins are precipitated after mixing and storage at

+8 °C overnight by adding 10 µl Dextranesulfate (10 %, w/v) and 50 µl CaCl₂ (1 mol/l) per ml serum.

8. The method as claimed in Claim 7, c h a r a c t e r i z e d in
5 that the obtained mixture is stored for 30-60 min. at +8 °C and then centrifuged at about 10 000 x g for about 10 min.
9. The method as claimed in Claim 8, c h a r a c t e r i z e d in
10 that the supernatant of the centrifuged mixture is diluted fivefold with water.
10. The method as claimed in Claim 9, c h a r a c t e r i z e d in that the diluted supernatant is injected to an HPLC.

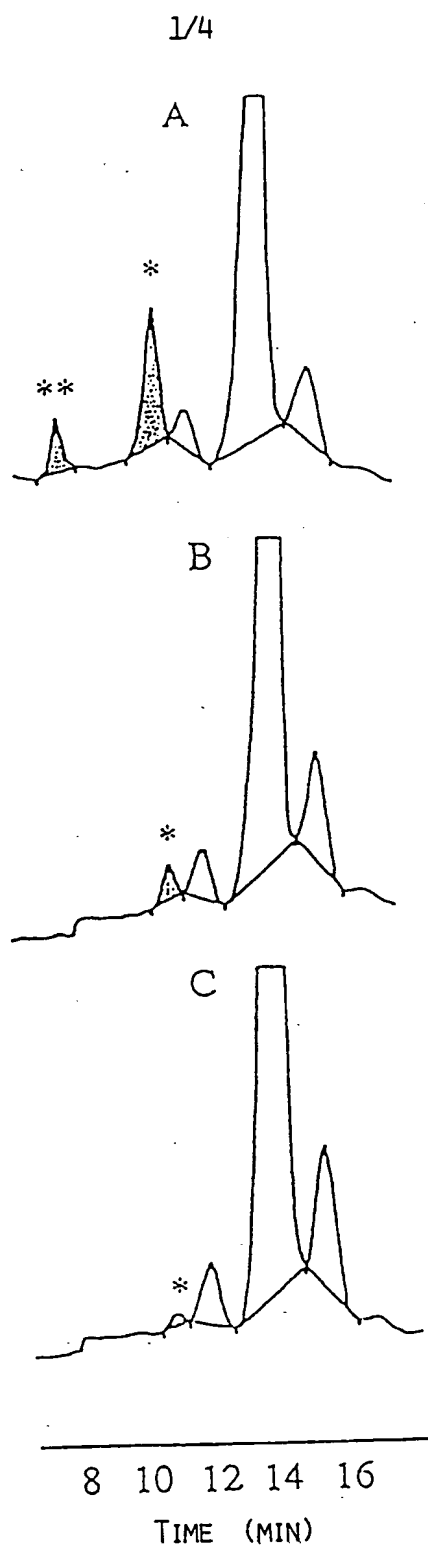


FIG 1

2/4

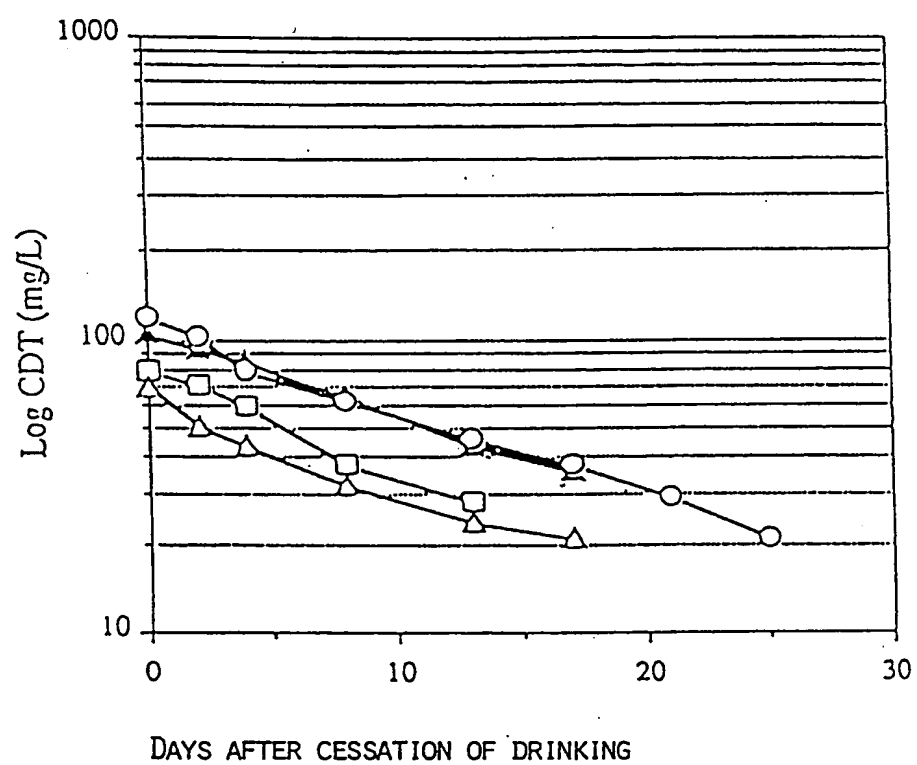


FIG 2

3/4

GRADIENT PROFILE

t (min)	A %	B %	C %
0	100	0	0
3.0	100	0	0
5.0	90	10	0
18.0	66	34	0
18.1	0	0	100
22.0	100	0	0
32.0	100	0	0

FIG 3

4/4

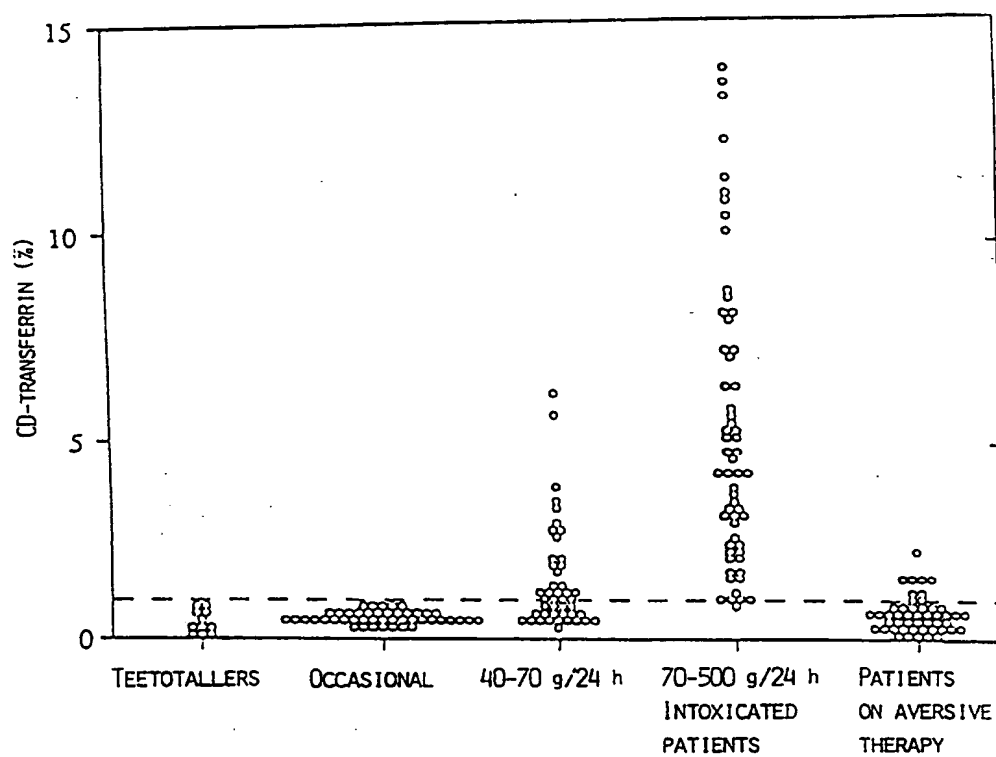


FIG 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 94/00684

A. CLASSIFICATION OF SUBJECT MATTER		
IPC6: G01N 33/68, C07K 14/79 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
MEDLINE, WPI		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	National Library of Medicine database, Medline, File Med 91, NLM Accession no. 92110905 Stibler H. et al: "Carbohydrate-deficient transferrin in serum: a new marker of potentially harmful alcohol consumption reviewed", & Clin Chem 1991 Dec; 37 (12):2029-37 --	1-10
A	National Library of Medicine database, Medline, File Med 85, NLM Accession no. 87251085, Pask-Hughes R.A. et al: "Characterisation and purification of some glycoproteins by high- performance liquid chromatography", & J Chromatogr 1987 Apr 29; 393(2):273-84 --	1-10
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
22 November 1994		23-11-1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer Carl Olof Gustafsson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 94/00684

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	National Library of Medicine database, Medline, File Med 85, NLM Accession no. 88047293, Petren S. et al: "Differences among five main forms of serum transferrin", & Alcohol Clin Exp Res 1987 Oct; 11(5):453-6 --	1
P,X	National Library of Medicine database, Medline, File Med. NLM Accession no. 94007163, Jeppsson J.O. et al: "Carbohydrate-deficient transferrin quantified by HPLC to determine heavy consumption of alcohol", & Clin Chem 1993 Oct; 39(10):2115-20 --	1-10
A	Dialog Information Services, File 155: Medline 66-90/May, accession no. 03616083, Medline accession no. 78250083, Hellsing K. et al: "Pre-treatment of serum samples for immunonephelo- metric analysis by precipitation with polyethylene glycol", & Scand J Clin Lab Invest Oct 1977, 37 (6) p529-36 --	7-10
A	WO, A1, 8503578 (PHARMACIA AB), 15 August 1985 (15.08.85), see page 5, second paragraph --	1-10
A	WO, A1, 9119983 (AXIS RESEARCH AS ET AL), 26 December 1991 (26.12.91), see pages 12-13 -- -----	1-10

INTERNATIONAL SEARCH REPORT
Information on patent family members

29/10/94

International application No.
PCT/SE 94/00684

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 8503578	15/08/85	AU-A- 3935185	27/08/85
		EP-A,B- 0172217	26/02/86
		SE-T3- 0172217	
		JP-B- 4031354	26/05/92
		JP-T- 61501111	29/05/86
		SE-B,C- 440699	12/08/85
		US-A- 4626355	02/12/86
<hr/>			
WO-A1- 9119983	26/12/91	AU-A- 8322891	07/01/92
		EP-A- 0535162	07/04/93
		HU-A- 65621	28/07/94
		JP-T- 6502912	31/03/94
		US-A- 5352616	04/10/94
<hr/>			